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Associations between Arsenic Species in Exfoliated Urothelial Cells and Prevalence of Diabetes among Residents of Chihuahua, Mexico

Jenna M. Currier,[1] María C. Ishida,[2] Carmen González-Horta,[2] Blanca Sánchez-Ramírez,[2] Lourdes Ballinas-Casarrubias,[2] Daniela S. Gutiérrez-Torres,[2] Roberto Hernández Cerón,[3] Damián Viniegra Morales,[3] Francisco A. Baeza Terrazas,[3] Luz M. Del Razo,[4] Gonzalo G. García-Vargas,[5] R. Jesse Saunders,[6] Zuzana Drobná,[6] Rebecca C. Fry,[7] Tomáš Matoušek,[8] John B. Buse,[9] Michelle A. Mendez,[6,10] Dana Loomis,[11] and Miroslav Stýblo[1,6]

[1]Curriculum in Toxicology, University of North Carolina, Chapel Hill, NC, USA; [2]Programa de Maestría en Ciencias en Biotecnología, Facultad de Ciencias Químicas, Universidad Autónoma de Chihuahua, Chihuahua, México; [3]Colegio de Médicos Cirujanos y Homeópatas del Estado de Chihuahua, A.C.; [4]Departamento de Toxicología, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, México Distrito Federal, México; [5]Facultad de Medicina, Universidad Juárez del Estado de Durango, Gómez Palacio, Durango, México; [6]Department of Nutrition and [7]Department of Environmental Sciences and Engineering, Gillings School of Global Public Health, University of North Carolina, Chapel Hill, North Carolina, USA; [8]Institute of Analytical Chemistry of the Academy of Sciences of the Czech Republic, v. v. i., Brno, Czech Republic; [9]School of Medicine, University of North Carolina, Chapel Hill, North Carolina, USA; [10]Carolina Population Center and Lineberger Cancer Center, University of North Carolina, Chapel Hill, North Carolina, USA; [11]International Agency for Research on Cancer, Monographs Section, Lyon Cedex, France

Address correspondence to Miroslav Stýblo, Department of Nutrition, CB# 7461, 135 Dauer Drive, Room 2302, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7461 USA. Telephone: (919) 966-5721. Fax: (919) 843-0776. E-mail: styblo@med.unc.edu

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Abstract

Background: A growing number of studies link chronic exposure to inorganic arsenic (iAs) with risk of diabetes. Many of these studies assessed iAs exposure by measuring arsenic (As) species in urine. However, this approach has been criticized because of uncertainties associated with renal function and urine dilution in diabetic individuals.

Objectives: Our goal was to examine associations between prevalence of diabetes and concentrations of As species in exfoliated urothelial cells (EUC) as an alternative to the measures of As in urine.

Methods: We measured concentrations of trivalent and pentavalent iAs, methyl-As (MAs), and dimethyl-As (DMAs) species in EUC from 374 residents of Chihuahua, Mexico, who were exposed to iAs in drinking water. We used fasting plasma glucose, glucose tolerance tests, and self-reported diabetes diagnoses or medication to identify diabetic participants. Associations between As species in EUC and diabetes were estimated using logistic and linear regression adjusting for age, sex, and body mass index.

Results: We found that interquartile range increases in trivalent, but not pentavalent As species in EUC were positively and significantly associated with diabetes, with OR of 1.57 (95% CI: 1.19, 2.07) for iAs^{III}, 1.63 (1.24 – 2.15) for MAs^{III}, and 1.31 (0.96 – 1.84) for $DMAs^{III}$. DMAs/MAs and DMAs/iAs ratios were negatively associated with diabetes (OR = 0.62; 95% CI: 0.47, 0.83 and OR = 0.72; 95% CI: 0.55, 0.96, respectively).

Conclusions: Our data suggest that uncertainties associated with measures of As species in urine may be avoided by using As species in EUC as markers of iAs exposure and metabolism, and provide additional support to previous findings suggesting that trivalent As species may be responsible for associations between diabetes and chronic iAs exposure.

Introduction

Arsenic (As), and specifically its inorganic forms (iAs), arsenite (iAs^{III}), and arsenate (iAs^{V}), are naturally occurring drinking water contaminants. Epidemiologic evidence (James et al. 2013; Kuo et al. 2013; Maull et al. 2012; Pan et al. 2013; Wang et al. 2014), including prospective studies (Kim et al. 2013) has linked chronic iAs exposure with diabetes mellitus. Several mechanisms by which iAs exposure can disrupt glucose homeostasis have been proposed (Maull et al. 2012). Trivalent iAs^{III} and the trivalent methylated arsenicals that are produced in the course of iAs metabolism, methylarsonite (MAs^{III}) and dimethylarsinite ($DMAs^{III}$), play key roles in these mechanisms (Douillet et al. 2013; Fu et al. 2010; Paul et al. 2007, 2008). However, assessing the association between these arsenicals and risk of diabetes in population studies has been a major challenge. This is because iAs^{III}, and particularly MAs^{III} and $DMAs^{III}$ are unstable in human urine, which has been traditionally used for assessment of iAs exposure and metabolism (Del Razo et al. 2011; Gong et al. 2001). An additional challenge is associated with quantification of iAs metabolites in urine, specifically with urinary creatinine as a factor adjusting for variation in urine dilution. Urinary creatinine is influenced by various factors, including age, sex, health status, ethnicity, body mass index, fat-free mass, and time of collection (Barr et al. 2005; Boeniger et al. 1993; Mahalingaiah et al. 2008). In addition, adjusting for creatinine may be inappropriate for individuals with compromised renal function, including people with diabetes (Jerums et al. 2010). More importantly, iAs exposure may lead to increased excretion of creatinine (Nermell et al. 2008). Thus, analysis of iAs metabolites in body fluids other than urine, or in cells or tissues, may provide a better measure of iAs exposure.

We have previously demonstrated the feasibility of As analysis in human cells, including exfoliated urothelial cells (EUC). In 2008, we used hydride generation (HG)-cryotrapping (CT)-

atomic absorption spectrometry (AAS) to measure concentrations of total iAs (iAs^{III+V}), MAs (MAs^{III+V}), and DMAs ($DMAs^{III+V}$) in EUC isolated from urine of residents of the Zimapan region in Mexico (Hernández-Zavala et al. 2008b). Because of small sample sizes, we could not perform the oxidation state specific analysis to distinguish between As^{III} and As^{V} species. However, we were able to detect and quantify both As^{III} and As^{V} species in cultured human urothelial cells treated *in vitro* with iAs. We have shown that both MAs^{III} and $DMAs^{III}$ are stable in these cells when stored at -80^{o}C (Currier et al. 2011a,b). To increase feasibility of the analysis of MAs^{III} and $DMAs^{III}$ in small numbers of EUC that are typically available from a spot urine sample, we have recently replaced AAS in the HG-CT-AAS system with inductively coupled plasma-mass spectrometer (ICP-MS). The newly developed HG-CT-ICP-MS proved to be suitable for the oxidation state specific analysis of As in EUC, providing superior detection limits and high reproducibility (Matoušek et al. 2013).

The goal of the present study was to determine concentrations of As^{III} and As^{V} species in EUC from individuals exposed to iAs in drinking water and to examine the association between As species in EUC and prevalence of diabetes.

Materials and Methods

The Chihuahua cohort

All procedures involving human subjects were approved by IRBs at the University of North Carolina at Chapel Hill (UNC) and Cinvestav-IPN. Individuals participating in the present study were among 1,163 men and women recruited for the Chihuahua cohort (Mexico); all study participants provided informed consent. This cohort was established between 2008 and 2013 to study chronic diseases associated with iAs exposure in drinking water. Only adults (≥ 18 years old) with at least 5 years of uninterrupted residency in the study area were recruited. Pregnant

women and participants who reported kidney or urinary tract infection were excluded because these conditions could affect the urinary pattern of iAs metabolites. Individuals with a potential for occupational exposure to As were also excluded. Data on residency, occupation, drinking water sources and consumption, smoking, use of alcohol, drugs or medication, and medical history were gathered at the time of enrollment using a questionnaire. Specific questions were asked about previous diagnosis of diabetes and use of anti-diabetic drugs. Samples of household tap water were collected for As analysis. The participants were then transported to Universidad Autónoma de Chihuahua to undergo a medical examination. Body weight and height were recorded and used to calculate the body mass index (BMI). A single spot urine sample was collected in sterile plastic cups and placed immediately on ice. Aliquots of urine samples were frozen and stored at -80^{o}C for speciation analysis of As; the rest was used for EUC isolation. A single sample of fasting venous blood was drawn followed by a standard oral glucose tolerance test (OGTT). Here, a sample of venous blood was drawn 2 hours after an oral load of 75 g glucose. All blood samples were placed on ice immediately after collection. Plasma was isolated from the fasting and 2-hour blood by centrifugation at 4^{o}C and frozen at -80^{o}C.

Isolation of EUC

EUC were isolated from individuals recruited between March 2011 and August 2012. A total of 466 individuals underwent medical examination during this period; 428 provided urine for EUC isolation. EUCs were isolated from freshly collected urine (100 mL/subject) by centrifugation at 4^{o}C. The cell pellet was washed with ice-cold phosphate-buffered saline (PBS), and again centrifuged. Cells were then resuspended in PBS, counted and checked for presence of bacteria, yeast, and red or white blood cells using a microscope. Only EUC free of microbial contamination and with less than 5% of total cell count represented by red or white blood cells

(from a total of 374 participants) were used in the present study. All cells other than bacteria, yeast, RBCs or WBCs were assumed, but not confirmed, to be EUC. EUC were stored at -80^{o}C and shipped along with the urine samples in dry ice to UNC once per month for As speciation analysis.

Diagnosis of diabetes

Glucose levels in fasting and 2-hour plasma samples were measured by Prestige 24i Chemistry Analyzer (Tokyo Boeki, Tokyo, Japan). The analyzer was calibrated prior to analysis, and reference human sera with normal and elevated glucose levels (Serodos and Serodos PLUS, Human Diagnostics Worldwide) were used for quality control. Study participants with fasting plasma glucose (FPG) ≥ 126 mg/dL or 2-hour plasma glucose (2HPG) ≥ 200 mg/dL, or with self-reported doctor's diagnosis or self-reported use of anti-diabetic medication were classified as diabetic.

Analyses of As in household water and urine

Concentrations of As in acid-digested water samples were determined in Cinvestav-IPN (Mexico City) using HG-CT-AAS (Del Razo et al. 2011). Urines were analyzed at UNC after storage at -80^{o}C for up to ~1 month, which is known to result in oxidation of MAs^{III} and $DMAs^{III}$ (Del Razo et al. 2011). Thus, only analysis of total iAs (iAs^{III+V}), MAs (MAs^{III+V}), and DMAs ($DMAs^{III+V}$) was performed using HG-CT-AAS (Hernández-Zavala et al. 2008a). A certified standard reference material, Arsenic Species in Frozen Human Urine (SRM 2669; National Institute of Standards and Technology) was used with every shipment to assure accuracy. The concentrations of As species measured by HG-CT-AAS in SRM 2669 ranged from 86.7 to 106.4% of the certified values: 90.3%-106.4% for iAs, 86.7%-96.4% for MAs, and 88.2%-99.0%

for DMAs. The limits of detection (LOD) using 200 μL of urine per sample were 0.05 ng As/mL for MAs or DMAs and 0.1 ng As/mL for iAs. Creatinine concentration in urine was determined by a colorimetric assay (Cayman Chemical Company, Ann Arbor, MI); specific gravity was measured using a digital Atago PAL refractometer (Atago USA, Bellevue, WA). It should be noted that the HG-CT-AAS cannot detect organic As species commonly found in seafood (e.g., arsenobetaine), and thus accounts for As species associated mainly with iAs exposure.

Analyses of As species in EUCs

As^{III} and As^{V} species in EUCs were analyzed at UNC using HG-CT-ICP-MS (Matoušek et al. 2013). Briefly, cell pellets were lysed in ice-cold deionized water. The trivalent species (As^{III}, MAs^{III}, and $DMAs^{III}$) were measured in an aliquot of cell lysate directly, without pretreatment. Another aliquot was treated with 2% cysteine and analyzed for total iAs (iAs^{III+V}), MAs (MAs^{III+V}), and DMAs ($DMAs^{III+V}$). The concentrations of iAs^{V}, MAs^{V}, and $DMAs^{V}$ were determined as a difference between As^{III+V} values obtained for cysteine-treated and As^{III} values from untreated sample aliquots. For As^{III} species concentrations below LOD, the values of LOD/√2 were used when calculating the corresponding As^{V} values. Calibration curves were generated using cysteine-treated pentavalent As standards (at least 98% pure) as previously described (Hernández-Zavala et al. 2008a). The instrumental LODs for As species analyzed by HG-CT-ICP-MS ranged from 0.04 to 2.0 pg As. The analyses of As species in EUC were performed by a researcher who was unaware of the individual's diabetes status or As concentrations in the corresponding urine and water samples.

Statistical analysis

Continuous variables were described using means and standard deviations (SD) or medians and interquartile ranges (the latter for non-normally distributed variables). Categorical variables were described using frequencies. For As species concentrations below LOD, the values of LOD/√2 were used for statistical analysis, including regression and descriptive analyses and to determine interquartile ranges (IQRs). The statistical significance of differences in characteristics of study participants with vs. without diabetes was assessed using Student's T tests. Associations between As species in EUC and urine were estimated using linear regression models with log-transformed (log_{10}) variables, as well as using Spearman correlations. Associations of diabetes with concentrations of As species in EUC and urine were estimated using logistic regression to estimate odds ratios (ORs) and 95% confidence intervals (CIs). To control for potential confounding, sex (as a categorical variable), and age and BMI (as continuous variables) were included *a priori* as covariates. We also used linear regression models adjusted for age, sex, and BMI to estimate associations of log_{10}-transformed FPG and 2HPG concentrations with concentrations of iAs metabolites and the sum of speciated As in urine. Age, sex, and BMI were included as covariates in these models. The linearity of the associations between As species and FPG/2HPG was assessed graphically and by linear regression using log_{10}-transformed values. Slopes were significantly non zero for all As species except pentavalent species in EUC. Unless otherwise specified, ORs, regression coefficients, and CIs are reported for a one IQR increment of exposure to facilitate comparison because of the different concentration ranges of As in EUCs and urine. Analyses of urinary metabolites of iAs were conducted both with and without urinary creatinine concentration or specific gravity adjustment. All statistical analyses were performed in Epi Info 7 version 1.0.6 (Centers for Disease Control and Prevention, Atlanta, GA) and graphical

representations were generated using GraphPad Instat software package (GraphPad Software Inc., San Diego, CA). Statistical significance was considered at the level of $p < 0.05$.

Results

Basic characteristics of the study population

The EUC samples free of microbial contamination and containing less than 5% of red or white blood cells were obtained from a total of 374 participants (252 women and 122 men). Over 17% of these participants (17.5% of women and 18% of men) were classified as diabetic (Table 1) based on the FPG or 2HPG value, or on the self-reported diabetes diagnosis or medication. Eleven participants that reported a previous diabetes diagnosis and/or taking anti-diabetic medication (25.5% of those classified as diabetic) had FPG and 2HPG values in the normal or prediabetic range (i.e., FPG < 126 and 2HPG < 200 mg glucose/dL). Approximately 29% of the participants were overweight and 41% were obese. The average age and BMI were significantly higher among diabetic compared with non-diabetic individuals. No statistically significant differences between diabetic and non-diabetic individuals were found in the average EUC count, As concentration in drinking water, or sum of As species in urine (expressed either as ng As/mL or ng As/mg of creatinine). The average specific gravity of urine from diabetic individuals was significantly higher than in non-diabetic individuals, but the difference between the two groups in the sum of As species in urine adjusted for specific gravity was not statistically significant. The average sum of As species was higher in EUC from non-diabetic as compared to diabetic participants, again; this difference was not statistically significant. Notably, basic characteristics of participants included in the present study and those of the entire Chihuahua cohort were very similar; however, the average age of the cohort was somewhat lower (Supplemental Material, Table S1).

Arsenic species in EUC and urine

The speciation analysis of As was performed in all 374 samples of EUC and in the corresponding urine samples (Table 2). Concentrations of all As^{III} and As^{V} species were above LOD in 94% of EUC samples. In urine, concentrations of iAs and MAs were below LOD in 15 (4%) and 2 (0.5%) samples, respectively. DMAs was detected and quantified in all urine samples. There were marked differences in As speciation profiles in EUC and in urine (Figure 1). The ratios of DMAs/MAs and MAs/iAs were lower in EUC as compared to urine. In urine, total DMAs (i.e., $DMAs^{III+V}$) was the predominant species, accounting on average for 76% of all As species with only 9% and 15% represented by iAs and MAs, respectively. In contrast, iAs^{III} and iAs^{V} were the major As species in EUC, representing on average 37% and 29% of speciated As. DMAs accounted for ~ 22% (2.4% for $DMAs^{III}$ and 19.2% for $DMAs^{V}$) and MAs for ~ 14% (MAs^{III} for 7.9% and MAs^{V} for 5.6%) of speciated As in EUC. In spite of the differences in As speciation profiles, statistically significant positive associations were found between the concentrations of individual As species in urine (not adjusted for creatinine) and in EUC (Supplemental Material, Figure S1). Notably, adjusting for creatinine had little or no effects on these associations (Supplemental Material, Table S2).

EUC counts and As speciation in EUC and urine according to gender

We found statistically significant differences in the numbers of EUC obtained from males and females. Cell counts in urine from males ranged from 450 to 2,128,000 cells/100 mL, compared with 1,800 to 9,717,000 cells/100 mL in samples from females. The mean EUC count (± SE) was 10 times higher in urine from women compared with men (529,258 ± 52,218/100 mL *vs.* 53,328 ± 18,782/100 mL). On average, EUC from females contained significantly less total speciated As than EUC from males: 40 ± 163 (SD) pg/10,000 cells vs. 309 ± 542 pg/10,000 cells

(Supplemental Material, Figure S2B). For both males and females, statistically significant negative associations were found between the cell count and the concentration of total speciated As in EUC (Supplemental Material, Figure S2). This association was stronger for males than females: β = -0.70 (95% CI: -0.82, -0.58; r^2 = 0.54) vs. β = -0.35 (95% CI: -0.46, -0.23; r^2 = 0.12). Gender related differences were also found in the composition of As species in both EUC and urine (Figure 2). iAs^{III} and MAs^{III} represented on average 43% and 10% of total speciated As in EUC from females, but only 18% and 4% in EUC from males (Figure 2A). On the other hand, the pentavalent As species, iAs^{V}, MAs^{V} and $DMAs^{V}$, accounted for greater proportions of As in EUC from males. Taken together, iAs species (iAs^{III} and iAs^{V}) represented a smaller portion and DMAs species ($DMAs^{III}$ and $DMAs^{V}$) a greater portion of total speciated As in male as compared to female EUC. On average, males excreted significantly more As in urine as MAs^{III+V} (17%) than did females (14%), but the percentage of $DMAs^{III+V}$ was smaller (73% vs. 78%) (Figure 2B). The DMAs/MAs ratio was smaller in male compared to female urines: 4.7 ± 1.84 vs. 7.0 ± 6.16 ($p < 0.0001$).

Associations of diabetes with As species in EUC and urine – logistic regression analysis

The logistic regression analysis was performed using two models. Model 1 included all diabetic individuals as classified by FPG, 2HPG, or self-reported diagnosis or medication (n = 66). For Model 2, only individuals with FPG ≥ 126 mg/dL or 2HPG ≥ 200 mg/dL were considered diabetic (n = 55); eleven individuals who reported diabetes diagnosis or medication but had both FPG < 126 mg/dL and 2HPG < 200 mg/dL were excluded. Both models were adjusted for age, sex and BMI. Using either model, we found diabetes to be significantly associated with iAs^{III} and MAs^{III} concentrations in EUC; more strongly in Model 2 with ORs for each IQR of 1.75 (95% CI: 1.29, 2.39) and 2.02 (95% CI: 1.48, 2.77), respectively (Figure 3; see Supplemental Material,

Table S3 for numeric data). Diabetes in both models was negatively associated with the ratios of DMAs/MAs and DMAs/iAs in EUCs. Here again, the associations were stronger in Model 2: OR = 0.53 (95% CI: 0.38, 0.73) and OR = 0.65 (95% CI: 0.48, 0.89), respectively. Diabetes was also positively associated with $DMAs^{III}$ and with sum of As species in EUC, but these associations were statistically significant only for Model 2 (OR = 1.49, 95% CI: 1.04, 2.13 and OR = 1.41, 95% CI: 1.01, 1.97, respectively). Diabetes was also positively associated with total iAs^{III+V} and MAs^{III+V}. A marginally significant association was found with iAs^{V}, but not with other As^{V} species. Notably, adjusting for cell count as a covariate in sensitivity analyses had no significant impact on the associations between As species in EUC and diabetes (data not shown). For example, ORs for Model 2 were 1.81 (95% CI: 1.33, 2.48) for iAs^{III}, 1.93 (95% CI: 1.42, 2.62) for MAs^{III}, and 1.42 (95% CI: 1.02, 1.97) for $DMAs^{III}$, thus practically matching the values obtained without the adjustment (see above).

In urine, total iAs, MAs, DMAs and DMAs/MAs ratio were all positively associated with diabetes; however, these associations were statistically significant only for the DMAs/MAs ratio in Model 1 (OR = 1.37, 95% CI: 1.03, 1.84) and total DMAs in Model 2 (OR = 1.34, 95% CI: 1.02, 1.76) (Figure 4; see Supplemental Material, Table S3 for numeric data). When adjusted for creatinine, total iAs, MAs, DMAs, and sum of As species in Model 2 were all significantly associated with diabetes. In contrast, after adjusting for specific gravity all OR values were close to zero. Unlike creatinine, specific gravity itself was positively associated with diabetes: OR = 1.32, 95% CI: 1.01, 1.71 (Model 1) and OR = 1.42, 95% CI: 1.07, 1.89 (Model 2).

Associations of diabetes with As species in EUC and urine – linear regression analysis

Linear regression analysis using logarithmically (log_{10}) transformed FPG and 2HPG values was performed to further characterize associations between diabetes and As species in EUC and urine

(Table 3). Both FPG and 2HPG were positively associated with the trivalent As species (iAs^{III}, MAs^{III}, $DMAs^{III}$) and with the sum of As^{III+V} species in EUC ($p < 0.01$), but were not significantly associated with the pentavalent As species in EUC. The ratios of DMAs/MAs and DMAs/iAs in EUCs were negatively associated with FPG and 2HPG ($p \leq 0.04$). Statistically significant ($p = 0.04$) and marginally significant ($p = 0.08$) positive associations were found between the MAs/iAs ratio and FPG and 2HPG, respectively. FPG and 2HPG also were significantly associated with each of the As^{III+V} species in urine, regardless of adjustment for creatinine or specific gravity. Here again, specific gravity, but not creatinine, was positively associated with both FPG and 2HPG concentrations ($p < 0.01$). Both FPG and 2HPG were also positively associated with the urinary DMAs/MAs ratio.

Discussion

The evidence linking chronic iAs exposure to diabetes was reviewed by a 2011 National Toxicology Program (NTP) workshop. This review concluded that existing data provide limited to sufficient support for an association of diabetes with high iAs exposures in drinking water (Maull et al. 2012). The workshop also discussed methods accounting for urine dilution when urinary As is used as indicator of iAs exposure. Because of uncertainties associated with effects of iAs exposure or disease on urinary creatinine levels, the workshop review recommended that both raw and adjusted values should be reported (Maull et al. 2012). We followed this recommendation in the present study. We found all As^{III+V} species in urine to be positively associated with diabetes classified by FPG or 2HPG. When adjusting for creatinine, these associations were statistically significant in Model 2; without the adjustment significant association was found only with urinary $DMAs^{III+V}$ (Figure 4; Supplemental Material, Table S3). Because of the uncertainty associated with urinary creatinine, adjusting for specific gravity has

been recommended as an alternative method (Nermell et al. 2008). However, adjusting for specific gravity may bias associations with diabetes because urine of diabetic individuals, as compared to healthy subjects, contains higher levels of albumin and glucose, resulting in higher specific gravity (Chadha et al. 2001; Voinescu et al. 2002). Indeed, we found a statistically significant positive association between diabetes and specific gravity and no associations between diabetes and urinary As species after adjusting for specific gravity.

The main goal of the present study was to examine associations between prevalent diabetes and As species in EUC as an alternative marker of iAs exposure and metabolism. Because cells provide a reducing environment, the toxic trivalent arsenicals are relatively stable even in samples stored for weeks (Currier et al. 2011a,b). Our results show that As species profiles in EUC do not reflect those in urine. Specifically, iAs species are the major species in EUC but represent only a small percentage of As found in urine, while DMAs is the major urinary metabolite. High iAs levels in EUC could be explained by formation of iAs-containing intra-cytoplasmic and intra-mitochondrial inclusions. These inclusions have recently been found in EUC from leukemia patients treated with arsenic trioxide (Wedel et al. 2013) and in bladder epithelium of mice exposed to iAs in drinking water (Dodmane et al., 2014). We also show that the concentrations and proportions As species in EUC significantly differ between men and women. EUC from male donors contained ~ 10 fold more total speciated As than EUC from female donors; however, iAs^{III} and MAs^{III} accounted for smaller fractions of As in male as compared to female EUC. These differences can probably be explained by different cell counts and cell types present in male and female urine. The vesical trigone area of an adult female bladder is particularly susceptible to squamous metaplasia (Fortin et al. 2010; Tyler et al. 1962), a non-cancerous change in the epithelial lining that is ultimately responsible for greater numbers

of cells in female urine. Female urine may also contain vaginal epithelial cells. However, the routine microscopy used in our study to examine EUC suspensions cannot reliably distinguish between epithelial cells of urothelial or squamous origin. Because of relatively small numbers of cells collected from spot urines, we could not apply more sophisticated techniques (e.g., immunocytochemistry) to characterize the types and origin of EUC. Thus, for the purpose of this study we defined all cells isolated from the bacteria- and yeast-free urine containing less than 5% of red or white blood cells as EUC.

We have previously reported that trivalent As species, particularly MAs^{III} and $DMAs^{III}$, inhibit insulin-dependent glucose uptake by adipocytes (Paul et al. 2007) and glucose stimulated insulin secretion by isolated pancreatic islets (Douillet et al. 2013). Our recent study in the Zimapan and Lagunera regions of Mexico indicated an association between the prevalence of diabetes and $DMAs^{III}$ concentration in urine (Del Razo et al. 2011). The present study found positive associations of diabetes with iAs^{III}, MAs^{III}, and $DMAs^{III}$ concentrations in EUC, thus providing additional evidence that trivalent metabolites of iAs play important roles in the diabetogenic effects of iAs exposure. Notably, DMAs/MAs and DMAs/iAs ratios in EUC were inversely associated with diabetes. While never before measured in EUC, the ratios of As species in urine have often been used in population studies to evaluate the body capacity to methylate iAs. Low DMAs/MAs and high MAs/iAs ratios in urine, as possible indicators of low methylation capacity, have been linked to increased risks of cancer and cardiovascular disease in populations chronically exposed to iAs exposure (Tseng 2007). In contrast, our study in Zimapan and Lagunera and the present study show that in case of diabetes, a high DMAs/MAs ratio in urine may be a risk factor (Del Razo et al. 2011).

In summary, our present study provides additional evidence for the association between diabetes and chronic exposure to iAs, and for the role of the trivalent metabolites of iAs in the diabetogenic effects of this exposure. We also show that the speciation analysis of As in EUC, which facilitates assessment of trivalent species and avoids the need for dilution adjustments, can be used as an alternative to analysis of urinary As species. It should be noted, however, that in this study the results obtained using the measures of As^{III} species in EUC were consistent with the results using measures of As^{III+V} species in urine after adjusting for creatinine. The associations between diabetes and As species in both EUC and urine were stronger when diabetes was classified only by FPG $\geq$ 126 or 2HPG $\geq$ 200 mg/dl, while disregarding self-reported diagnosis or medication. This is consistent with a recent report from an American Indian cohort where an association between iAs exposure and diabetes was observed only among participants with poor diabetes control as indicated by elevated levels of glycated hemoglobin (Gribble et al. 2012). Thus, using clinical indicators as compared to existing diabetes diagnosis may be required to better characterize the association of iAs exposure with prevalent or incident diabetes. The cross-sectional design, which cannot provide information on causality of the observed associations, and a modest sample size are the main limitations of the present study. Prospective studies in larger populations exposed to iAs, including the Chihuahua cohort, are needed to determine whether trivalent As species in EUC can be used to identify individuals or subpopulations who are at risk of developing diabetes as a result of chronic exposure to iAs.

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Table 1. Basic characteristics of the study participants [mean ± SD or n (%)].

Characteristics	All participants	Diabetic[a]	Non-diabetic
All participants	374 (100)	66 (17.6)	308 (82.4)
Females	252 (67.4)	44 (17.5)	208 (82.5)
Males	122 (32.6)	22 (18.0)	100 (82.0)
Age (years)	49.2 ± 15.6	56 ± 12.0*	48 ± 16.0*
As in drinking water (ppb)	55.2 ± 52.8	60.0 ± 50.9	54.1 ± 53.2
BMI	29.2 ± 6.1	30.8 ± 5.4*	28.9 ± 6.2*
26 ≤ BMI < 30	108 (29)	19 (29)	79 (26)
BMI ≥ 30	155 (41)	31 (47)	124 (40)
FPG (mg/dL)	95.9 ± 39.5	155.7 ± 62.8*	83.2 ± 12.1*
2HPG (mg/dL)	118.6 ± 60.4	204.9 ± 86.0*	100.4 ± 31.1*
FPG ≥ 126 mg/dL	38 (10.2)	38 (57.6)	0 (0)
2HPG ≥ 200 mg/dL	33 (8.8)	33 (50.0)	0 (0)
Self-reported diabetes diagnosis	43 (11.5)	43 (65.2)	0 (0)
Self-reported diabetes medication	30 (8.0)	30 (45.5)	0 (0)
EUC count in 100 ml urine[b]	374008 ± 726387	352165 ± 468388	378689 ± 771036
Sum of As species[c] in EUC (pg As/10,000 cells)	127.7 ± 359.6	90.3 ± 202.1	135.7 ± 384.8
Sum of As species in urine (ng As/mL)	73.9 ± 73.2	82.0 ± 74.9	72.1 ± 72.9
Creatinine concentration in urine (mg/dL)	129.2 ± 90.2	127.1 ± 85.7	129.6 ± 91.3
Sum of As species in urine normalized for creatinine (ng As/mg creatinine)	65.7 ± 66.5	73.1 ± 77.9	64.2 ± 63.8
Specific gravity of urine	1.014 ± 0.007	1.017 ± 0.008*	1.014 ± 0.007*
Sum of As species in urine normalized for specific gravity (ng As/specific gravity unit)	109.7 ± 101.8	92.6 ± 62	113.4 ± 108.2

[a]Diabetic individuals were classified by either FPG ≥ 126 mg/dL, 2HPG ≥ 200 mg/dL, self-report of doctor diagnosis or use of medication for treatment of diabetes. [b]Neither white or red blood cells were included in the cell counts. [c]Sum of As species = iAs^{V} + iAs^{III} + MAs^{V} + MAs^{III} + $DMAs^{V}$ + $DMAs^{III}$.

*For continuous variables, a statistically significant differences between participants with and without diabetes by Student's t-test, $p < 0.05$.

Table 2. The concentrations of As species in EUC and urine.

As species	Min.	25th percentile	Median	75th percentile	Max.	IQR	Mean ± SD
EUC (pg As/10,000 cells)							
iAs^{III}	<LOD[a]	2.08	8.18	17.69	1807	15.61	24.06 ± 103.0
MAs^{III}	<LOD	0.45	1.78	4.04	151.7	3.59	4.17 ± 11.22
$DMAs^{III}$	<LOD	0.16	0.41	1.54	141.3	1.38	2.73 ± 9.58
iAs^{V}	<LOD	1.27	4.53	22.66	728.7	21.39	34.79 ± 86.26
MAs^{V}	<LOD	0.19	0.85	5.08	776.2	4.89	13.11 ± 53.48
$DMAs^{V}$	<LOD	0.66	1.90	13.82	2303	13.16	49.18 ± 200.7
iAs^{III+V}	0.36	6.35	17.13	41.11	2148	34.76	58.52 ± 155.2
MAs^{III+V}	0.04	1.12	3.14	9.84	803.3	8.72	17.25 ± 57.87
$DMAs^{III+V}$	0.04	0.88	2.41	15.83	2366	14.95	51.91 ± 208.9
Sum of As species[b]	0.78	10.05	25.50	76.3	3773	66.25	127.7 ± 359.6
MAs/iAs	0.01	0.15	0.2	0.28	3.63	0.13	0.26 ± 0.28
DMAs/MAs	0.04	0.55	1.10	2.90	51.47	2.35	2.15 ± 3.64
DMAs/iAs	0.004	0.10	0.20	0.52	35.0	0.42	0.77 ± 2.5
(MAs+DMAs)/iAs	0.02	0.28	0.40	0.79	35.63	0.51	1.03 ± 2.68
Urine (ng As/mL)							
iAs^{III+V}	<LOD	0.98	4.62	10.21	119.2	9.23	7.3 ± 10.3
MAs^{III+V}	<LOD	2.23	7.34	15.97	131.1	13.74	11.1 ± 12.9
$DMAs^{III+V}$	0.36	13.05	40.46	82.72	307.2	69.67	55.42 ± 53.8
Sum of As species	0.52	17.00	53.50	108.4	492.5	91.40	73.87 ± 73.24
MAs/iAs	0.10	1.28	1.64	2.11	199.4	0.83	4.51 ± 18.1
DMAs/MAs	1.73	4.09	5.19	7.06	86.2	2.97	6.25 ± 5.27
DMAs/iAs	0.41	6.57	9.25	12.43	2117	5.86	29.1 ± 142.3
(MAs+DMAs)/iAs	0.51	8.07	10.94	14.47	2317	6.40	33.58 ± 159.6

[a]The minimum values were below the limit of detection (LOD) for iAs^{III} (n = 3), MAs^{III} (n = 13), $DMAs^{III}$ (n = 19), iAs^{V} (n = 6), MAs^{V} (n = 21), or $DMAs^{V}$ (n = 3) in EUC, and for iAs^{III+V} (n = 15) or MAs^{III+V} (n = 2) in urine. [b]Sum of As species = iAs^{V} + iAs^{III} + MAs^{V} + MAs^{III} + $DMAs^{V}$ + $DMAs^{III}$.

Table 3. Associations of FPG[a] and 2HPG[b] with As species in EUCs and urine (adjusted for age, sex, and BMI).

As species	FPG: β (95% CI)[c]	FPG: *p*[d]	FPG: r^2	2HPG: β (95% CI)	2HPG: *p*[c]	2HPG: r^2
EUC						
iAs^{III}	0.056 (0.038, 0.074)	<0.01	0.16	0.042 (0.024, 0.060)	<0.01	0.14
MAs^{III}	0.062 (0.044, 0.080)	<0.01	0.17	0.050 (0.032, 0.068)	<0.01	0.15
$DMAs^{III}$	0.050 (0.028, 0.072)	<0.01	0.12	0.039 (0.017, 0.061)	<0.01	0.12
iAs^{V}	0.013 (-0.005, 0.031)	0.15	0.07	0.007 (-0.011, 0.025)	0.45	0.09
MAs^{V}	0.015 (-0.001, 0.031)	0.08	0.08	0.010 (-0.006, 0.026)	0.21	0.09
$DMAs^{V}$	0.010 (-0.008, 0.028)	0.25	0.07	0.007 (-0.011, 0.025)	0.40	0.09
iAs^{III+V}	0.026 (0.012, 0.040)	<0.01	0.11	0.022 (0.008, 0.036)	<0.01	0.11
MAs^{III+V}	0.035 (0.021, 0.049)	<0.01	0.13	0.029 (0.015, 0.043)	<0.01	0.13
$DMAs^{III+V}$	0.015 (-0.001, 0.031)	0.06	0.08	0.014 (-0.002, 0.030)	0.08	0.09
Sum of As species	0.045 (0.023, 0.067)	<0.01	0.11	0.035 (0.013, 0.057)	<0.01	0.11
MAs/iAs	0.046 (0.003, 0.089)	0.04	0.08	0.039 (-0.004, 0.082)	0.08	0.09
DMAs/MAs	-0.072 (-0.101, -0.043)	<0.01	0.12	-0.057 (-0.088, -0.026)	<0.01	0.12
DMAs/iAs	-0.032 (-0.057, -0.007)	0.01	0.08	-0.033 (-0.064, -0.002)	0.04	0.10
(DMAs+MAs)/iAs	-0.014 (-0.045, 0.017)	0.40	0.07	-0.009 (-0.042, 0.024)	0.60	0.09
Urine (unadjusted)						
iAs^{III+V}	0.048 (0.030, 0.066)	<0.01	0.13	0.043 (0.025, 0.061)	<0.01	0.14
MAs^{III+V}	0.062 (0.042, 0.082)	<0.01	0.15	0.045 (0.023, 0.067)	<0.01	0.13
$DMAs^{III+V}$	0.078 (0.056, 0.100)	<0.01	0.18	0.061 (0.039, 0.083)	<0.01	0.15
Sum of As species	0.076 (0.054, 0.098)	<0.01	0.17	0.059 (0.037, 0.081)	<0.01	0.15
MAs/iAs	-0.001 (-0.038, 0.036)	0.95	0.02	-0.032 (-0.069, 0.005)	0.05	0.09
DMAs/MAs	0.072 (0.001, 0.143)	0.05	0.08	0.100 (0.029, 0.171)	<0.01	0.11
DMAs/iAs	0.016 (-0.019, 0.051)	0.37	0.07	-0.005 (-0.040, 0.030)	0.80	0.09
(DMAs+MAs)/iAs	0.014 (-0.021, 0.049)	0.45	0.07	-0.009 (-0.044, 0.026)	0.62	0.09
Creatinine	0.033 (-0.006, 0.072)	0.09	0.07	0.005 (-0.034, 0.044)	0.79	0.09
Specific gravity	10.314 (6.257, 14.371)	<0.01	0.13	5.995 (1.820, 10.170)	<0.01	0.11
Urine (creatinine adjusted)						
iAs^{III+V}	0.052 (0.030, 0.074)	<0.01	0.12	0.053 (0.031, 0.075)	<0.01	0.15
MAs^{III+V}	0.072 (0.048, 0.096)	<0.01	0.15	0.060 (0.035, 0.085)	<0.01	0.14
$DMAs^{III+V}$	0.093 (0.032, 0.154)	<0.01	0.18	0.083 (0.058, 0.108)	<0.01	0.18
Sum of As species	0.091 (0.066, 0.116)	<0.01	0.18	0.081 (0.056, 0.106)	<0.01	0.17

As species	FPG: β (95% CI)[c]	FPG: *p*[d]	FPG: r^2	2HPG: β (95% CI)	2HPG: *p*[c]	2HPG: r^2
Urine (specific gravity adjusted)						
iAs^{III+V}	0.040 (0.018, 0.062)	<0.01	0.10	0.042 (0.020, 0.064)	<0.01	0.13
MAs^{III+V}	0.055 (0.031, 0.079)	<0.01	0.12	0.042 (0.020, 0.064)	<0.01	0.13
$DMAs^{III+V}$	0.073 (0.048, 0.098)	<0.01	0.14	0.065 (0.040, 0.090)	<0.01	0.14
Sum of As species	0.070 (0.045, 0.095)	<0.01	0.14	0.062 (0.037, 0.087)	<0.01	0.14

[a]FPG, fasting plasma glucose. [b]2HPG, 2-hour plasma glucose determined by glucose tolerance test; log-transformed (log_{10}) FPG and 2HPG values were used in the linear regression analysis. [c]Regression coefficient (β) and 95% confidence interval (95% CI) are standardized to an increment of one inter-quartile range. [d]*p*-value for test of β = 0; linear regression model adjusted for age, sex, and BMI.

Figure legends

Figure 1. The composition of As species in EUC and urine. Each bar represents mean and SD values (n = 374). *Differences between EUC and urine are statistically significant ($p < 0.05$) based on one-way ANOVA.

Figure 2 Differences in the composition of As species in EUC (A) and urine (B) collected from male and female study participants. Each bar represents mean and SD values (n = 252 for women; n = 122 for men). *Differences between men and women are statistically significant ($p < 0.05$) as determined by one-way ANOVA with Bonferroni's posttest.

Figure 3. Association of diabetes with As species in EUC. Model 1, diabetes is classified by either FPG ≥ 126 mg/dL, 2HPG ≥ 200 mg/dL, self-report of doctor diagnosis or use of medication for treatment of diabetes; Model 2, diabetes is classified only by FPG ≥ 126 mg/dL or 2HPG ≥ 200 mg/dL. Odds ratio (OR) and 95% confidence interval (CI) are standardized to an increment of one inter-quartile range (IQR) and adjusted for age, sex, and BMI. IQRs for each As specie and species ratio are indicated in Table 2. Numeric data are provided in Supplemental Material, Table S3. *The association is statistically significant ($p < 0.05$).

Figure 4. Association of diabetes with As species in urine, urine creatinine and specific gravity. Arsenic species are either unadjusted (A) or adjusted for creatinine (B) or specific gravity (C). Model 1, diabetes is classified by either FPG ≥ 126 mg/dL, 2HPG ≥ 200 mg/dL, self-report of doctor diagnosis or use of medication for treatment of diabetes; Model 2, diabetes is classified only by FPG ≥ 126 mg/dL or 2HPG ≥ 200 mg/dL. Odds ratio (OR) and 95% confidence interval (CI) are standardized to an increment of one inter-quartile range (IQR) and adjusted for age, sex, and BMI. IQRs for each As specie and species ratio are indicated in Table 2. Numeric data are provided in Supplemental Material, Table S3. *The association is statistically significant ($p < 0.05$).

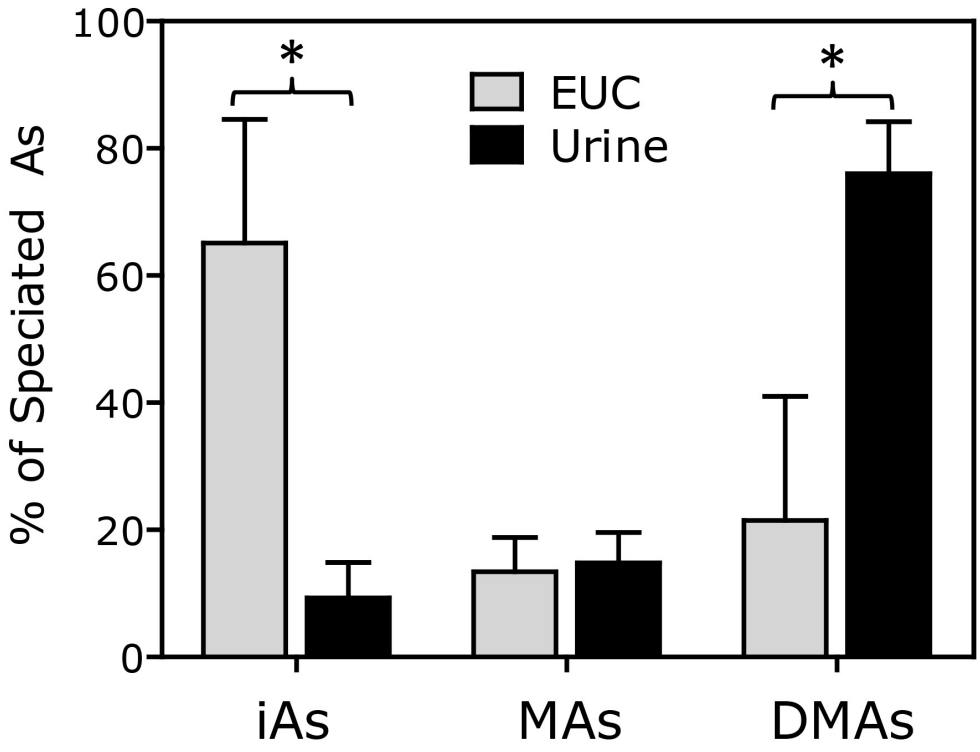
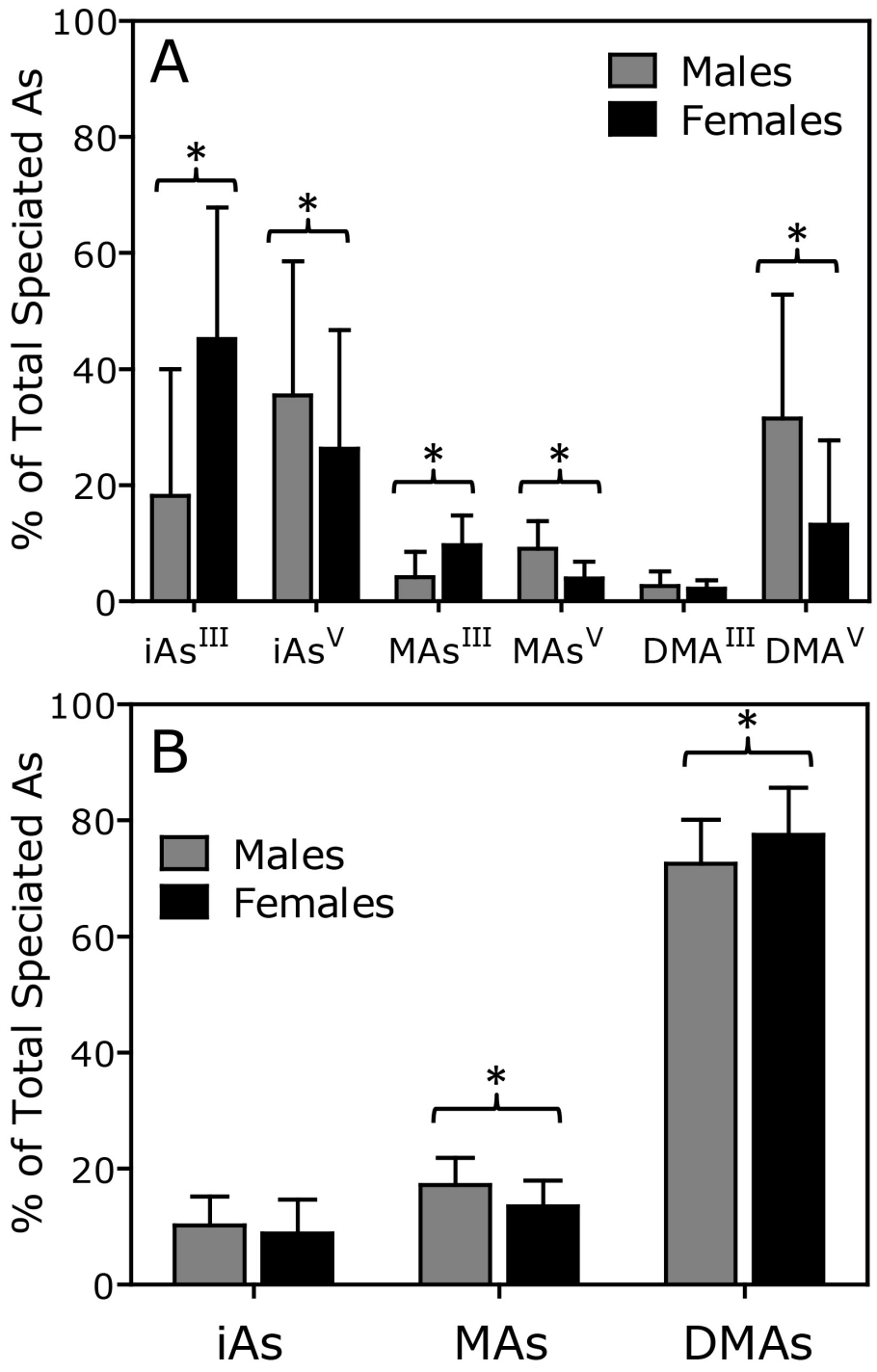


Figure 1


A
Males
Females
% of Total Speciated As
100
80
60
40
20
0
*
iAs III
iAs V
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MAs V
DMA III
DMA V
B
Males
Females
% of Total Speciated As
iAs
MAs
DMAs

Figure 2

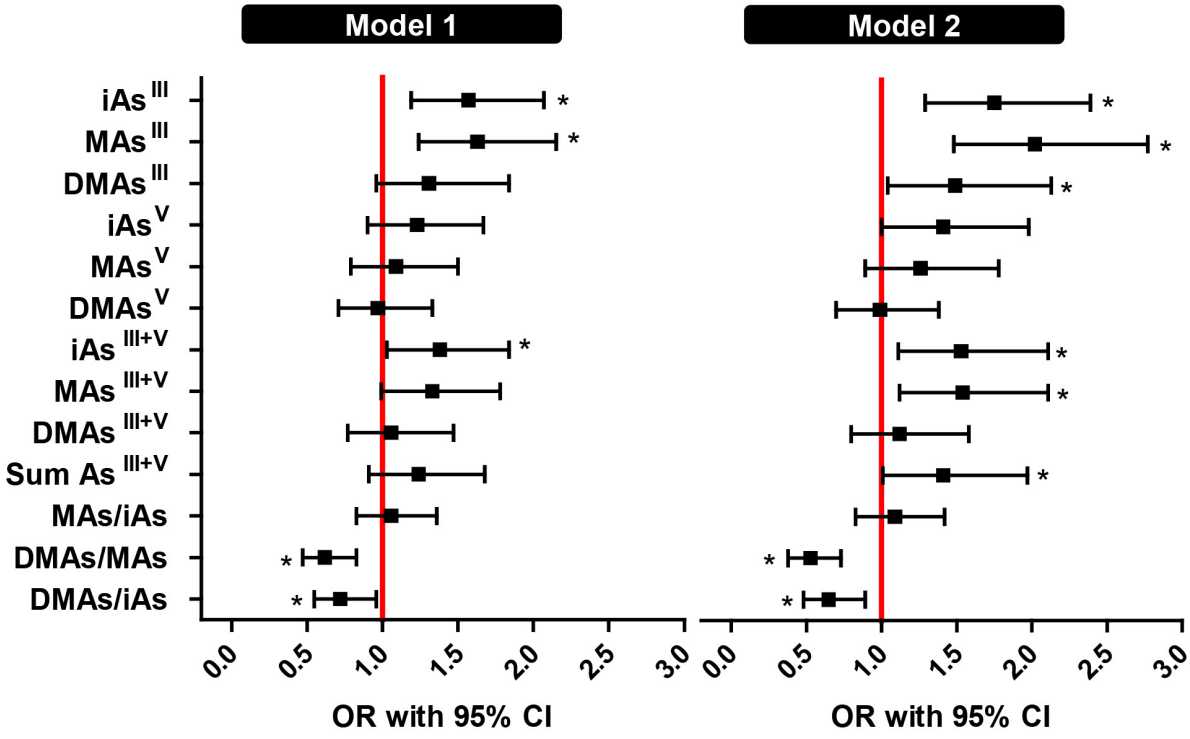
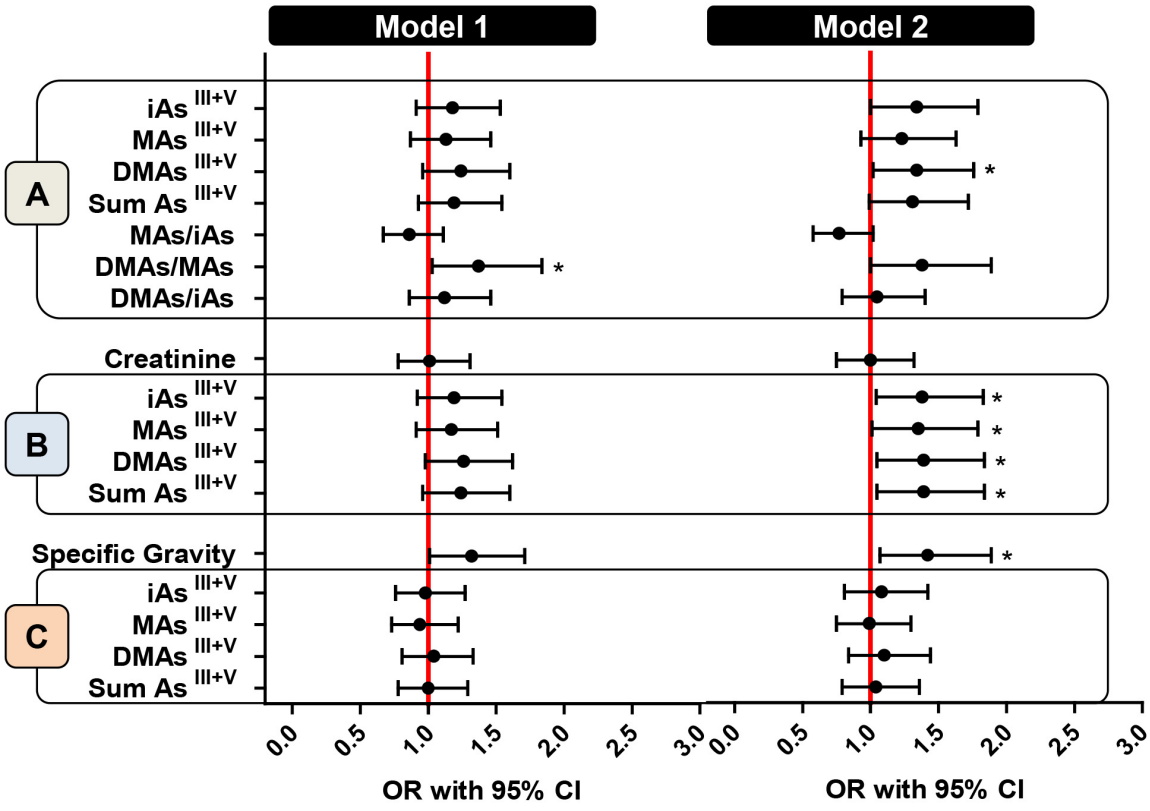

Model 1
Model 2
iAs III
MAs III
DMAs III
iAs V
MAs V
DMAs V
iAs III+V
MAs III+V
DMAs III+V
Sum As III+V
MAs/iAs
DMAs/MAs
DMAs/iAs
0.0
0.5
1.0
1.5
2.0
2.5
3.0
OR with 95% CI
OR with 95% CI

Figure 3


Model 1
Model 2
A
iAs III+V
MAs III+V
DMAs III+V
Sum As III+V
MAs/iAs
DMAs/MAs
DMAs/iAs
B
Creatinine
iAs III+V
MAs III+V
DMAs III+V
Sum As III+V
C
Specific Gravity
iAs III+V
MAs III+V
DMAs III+V
Sum As III+V
0.0
0.5
1.0
1.5
2.0
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OR with 95% CI
OR with 95% CI

Figure 4